2349 CHAPTER 5

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The Lipids of Milk: Deterioration

PART I RANCIDITY

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Market milk and some products manufactured from milk at times possess a flavor described as rancid. This term as used in the dairy industry denotes implicitly the flavor due to the presence of free fatty acids, more notably the lower volatile acids, hydrolytically cleaved from milkfat under the catalytic influence of the lipases normally present in milk.

The development of rancid flavor in milk and some of its products is usually undesirable and detracts from their market value. Indeed, the intensity of the flavor may reach such a degree as to render the product unsalable. In contrast, the popularity of certain dairy products and some confectionery items containing milk as one of the ingredients is thought to be due, at least in part, to the proper intensity of the rancid flavor. Hence, a knowledge of the factors involved in the development of rancidity is of great practical importance to several industries.

The literature on the subject is quite large. The present review has been limited to milk, but good reviews on this, other dairy products and on microorganisms are available.[9,58,132,133,304]

Terminology

Lipases are members of the wide class of enzymes catalyzing the hydrolysis of various esters, i.e., the esterases. It should be kept in mind that included in this class are the *esterases proper*, enzymes which split the simple, common, wholly organic esters such as ethyl acetate. Differentiation, therefore, between lipases and esterases proper is made on the basis of their relative preferential specificity. The natural substrates for lipases are oils and fats, that is, triglycerides of the fatty acids, whereas *esterases proper* act on simple

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esters. Both groups of enzymes are unusually nonspecific in their action, and the nature of either the fatty acid or alcohol residue has at most a secondary effect, influencing only the *rate* of hydrolysis of the ester.

Lipolysis is the term usually applied to the enzymatically catalyzed cleavage of triglycerides. Esterolysis is sometimes used as an all-inclusive term in non-dairy literature. However, this term is ostensibly general, and to avoid confusion will not be used in this discussion.

The term "enzyme system" has been proposed to indicate multiple enzymes.[145] It has been suggested that the term "lipase system" be used in dairy literature to express the lipase multiplicity in milk.[91] This expression will be adopted here and will be used synonymously with "lipases" and "lipolytic enzymes."

Terminology which implies the existence in milk of specific lipases, such as, for example, "tributyrinase," is inconsistent with the definition of lipases given above. This has unfortunately been a too common practice in milk lipase studies, and should, of course, be avoided since it has not been shown conclusively that enzymes present in milk are absolutely specific for a given substrate.

THE MILK LIPASE SYSTEM

Origin

Although of only theoretical interest as far as dairy technology is concerned, the fact that enzymes are produced and elaborated by living cells has prompted some investigation concerning the origin of the milk lipase system. Enzymes catalyze both the breakdown and synthesis of the substrate. Nothing is known about the milk lipase system insofar as its capacity to synthesize triglycerides is concerned. Whether the lipase system in milk is the same as that involved in milkfat synthesis in the udder is purely a matter of debate. The mammary gland preparations of Virtanen[359] had some ability to hydrolyze milkfat, but showed greater activity toward the simple ester, ethyl butyrate. However, Kleiner and Tauber[186] were unable to demonstrate esterase activity in mammary gland extracts. Kelly[176] found that the mammary glands of pregnant cows were more lipolytically active than those from nonpregnant cows. Heyndrickx and Peeters[138] reported that the ratio of lipase activity in the lymph of the udder, blood plasma, and milk was 1.0:1.9:1.3. It is of interest in this line of investigation to note that Morton[225–227] has shown that milk phosphatase is derived from mammary gland microsomes which are released into the milk during the normal secretory process.

Bovine blood serum is lipolytically active, but cows producing milk which goes rancid quickly do not have sera more lipolytically active than those producing normal milk.[276] Leucocytes, which are present in large numbers in milk and are especially high in mastitic milk, are the source of milk catalase, but are apparently not responsible for the elaboration of milk lipases.[233] The parenchyma cells of the mammary gland of women was considered by Beumer[21] to be the source of human milk lipase.

The lipases of milk are apparently inactive in the udder and at the time of milking.[132] Milk always contains some unesterified fatty acids when drawn,[345] but these may be left over from the metabolic pool. It will become evident later, that the milk lipase system is unusually slow-acting unless some physical or thermal manipulation is employed. This may account for the inactivity in the udder, but no experiments have been designed to substantiate this.

Spontaneous Vs. Induced Rancidity

Individual cows maintained under identical conditions seem to vary markedly in the susceptibility of their milk toward rancidity.[173,191] Numerous investigators have associated an increased incidence of rancidity with advanced lactation, particularly during long lactation periods.[5,12,39,64,86,139,154,188,243,280] However, published reports have failed to show any direct correlation between late lactation and rancidity.[136,219,262,282,364] Guthrie and Herrington[118] and Tarassuk *et al.*[340] have indicated that the occurrence of mastitis may be more important than late lactation in rancidity problems. Bachmann[12] acknowledges that late lactation milk is susceptible to rancidity, but also indicates that hormonal disturbances are a factor. Furthermore, he states the two types of rancid milks differ in that rancidity arising from late lactation appears to be due to a reduction in the protection of the fat against the enzymes, whereas rancidity originating from hormonal disturbances is due to an increase in enzyme concentration.

The cow's feed has also been shown to be an important practical factor in influencing the susceptibility of milk to rancidity.[5,7,164,334] Feeding experiments and practical observations have demonstrated that green pasture decreases, and dry feed increases, the occurrence of rancidity.

The number of cows producing milk which easily goes rancid reaches a maximum in late fall and then decreases through January, February, and March.[139,362]

Regardless of farm practices or of the physiological factors in-

volved, the fact remains that from 2 to 22% of cows in a herd produce milk which becomes rancid quickly.[139] Milk which inherently possesses the quality of high susceptibility toward rancidity has been variously termed "naturally rancid milk,"[54] "bitter milk of advanced lactation,"[243] "naturally active" or "naturally lipolytically active,"[201,324,326,329] "normally active"[345] and "spontaneous."[326,329] The latter name has come into favor during the last 20 years. These various designations were introduced in an effort to distinguish such milk from nonspontaneous or "normal" milk.

As was mentioned earlier, lipolysis in freshly drawn milk normally proceeds at a very slow rate, even upon prolonged incubation, unless the proper thermal or mechanical treatment is applied. Such manipulation, of course, is always carried out in practice since raw, warm milk is never consumed on the market. It is through these necessary practices that lipolysis in milk is accelerated. As a consequence milk may be made rancid either accidentally or deliberately. The so-called spontaneous type of milk needs no treatment other than cooling when drawn or shortly after milking to between 20° and 15°C. to hasten lipolysis.[336] Once the milk has been cooled, lipolysis is not materially affected whether the milk is aged in the cold or rewarmed immediately to 20°, 30°, or 37°C., and aged at those temperatures. On the other hand, lipolysis in nonspontaneous or normal milk is not accelerated to the same degree by cooling and aging. Additional thermal manipulation or physical processing such as homogenization or shaking is needed to speed up lipolysis. These will be discussed later in more detail.

The reason that rancid milk is not more predominant in market milk in view of what has just been said, is due to the fortuitous fact that spontaneous rancidity can be prevented or reduced by mixing such milk within one hour after milking with 4 to 5 times its volume of nonspontaneous (normal) milk.[329] Since usually only one out of five cows in a herd produce spontaneous milk, this defect is thus automatically eliminated. It is clear, however, that farmers with 1 or 2 cows are likely to encounter spontaneously rancid milk sometime during the lactation period.

There has been no published research on the mechanism involved in the inhibition of lipolysis in spontaneous milk by dilution with nonspontaneous milk. The most obvious interpretation is that nonspontaneous milk contains a labile inhibitor of the lipase involved in spontaneous rancidity. The dilution of nonspontaneous milk which has been activated by thermal or physical manipulation does not diminish activity.[108,205,224]

Number, Nature and Distribution of Lipases in Milk

Sufficient evidence exists now to support the view that more than one lipase is present in all raw milk. This was first suggested by Herrington and Krukovsky on the basis of the inhibition of lipolysis by formalin. In this instance[135] and in subsequent studies[136,283] low concentrations of formalin were as effective as high concentrations in the degree of inhibition produced. These observations led to the proposal[135] that a formalin-sensitive and a formalin-tolerant lipase exist in milk. Although this supposition was more speculative than convincing, it served to stimulate research in this direction which resulted in the accumulation of considerable fundamental knowledge concerning development of rancidity.

Schwartz[298] presented kinetic data, and conducted more detailed studies on formalin inhibition,[299] the results of which were interpreted in favor of the presence of more than one lipase in raw skim-milk. Albrecht and Jaynes[2] and Nelson[232] obtained multiple pH optima on tributyrin with their enzyme sources. The use of kinetic data as a means of showing multiple lipase activity was criticized as being too presumptious, especially when carried out in a two-phase system.[90]

Evidence to substantiate the contention that milk contains a lipase system was considerably strengthened by the investigations of Frankel and Tarassuk[89-91] and Tarassuk and Frankel.[328] They showed that the relative specificity of the milk lipase system toward different substrates differed between individual milks. If only one nonspecific lipase was present in milk, the relative lipolytic activity observed in different milks would be expected to be constant. This is based on the valid assumption that the relative enzyme activity in a system is independent of enzyme concentration, provided that the substrate is always present in sufficient concentration to saturate the enzymes. Treatment with acid, alkali, trypsin, light, oxidizing agents, and formaldehyde was later utilized to effect a differential inactivation of the enzymes involved.[90]

Indirect evidence for the multiplicity of the milk lipase system is also suggested by pH-activity curves obtained on milkfat and on tributyrin. Invariably, more activity is obtained on milkfat in the pH range 6.0 to 7.5 than is noticed when tributyrin is utilized as substrate. At the normal pH of milk (about 6.6) there is no perceptible enzymatic hydrolysis of tributyrin when short ($^1/_2$ to 1 hr.) incubation periods are employed.[60,232,260] Whole milk, however, goes rancid at its normal pH, and relatively good activity is obtained within one

hour in homogenized milk.[296,298,299] The implications here are, of course, that an enzyme is operating at the normal pH of milk which is more specific for milkfat than for tributyrin.[296]

The combined investigations on the heterogeneity of the milk lipase system have not established the exact number of lipases in milk. Milk contains a lipoprotein lipase[271] similar to that found in mammalian adipose and heart tissue. The enzyme is highly specific for lipoproteins and probably is not involved in rancidity development to any great extent. Milk is reported by Forster and his group[78,80–83] to contain at least three simple esterases designated A, B, and C. The B-esterase has been partially purified[123,124,223] and has been shown to be active on milkfat.[158] Besides these enzymes it may be stated with some assurance that all cow milk contains a minimum of two true lipolytic enzymes. One of these has been termed the "plasma lipase," the other the "membrane lipase."[328] Evidence has been offered to show that when freshly drawn milk is cooled, irreversible adsorption of the membrane lipase onto the material enveloping the fat globules takes place. The other (plasma) lipase remains in the skimmilk and is intimately associated with the caseinate fraction.[328] The phenomenon of irreversible adsorption of a lipase upon the fat globule membrane had been suggested earlier.[330] However, this proposal was not generally accepted at the time primarily for want of sufficient direct experimental evidence.

Following upon the data accumulated in favor of a plasma and membrane lipase, Tarassuk and Frankel[328] attempted to explain the phenomenon of spontaneous milk on the basis of concentration of membrane lipase in milk. According to them the concentration of membrane lipase varies from very low in nonspontaneous (normal) milk, i.e., milk which will not develop perceptible rancidity on cooling and aging for over 48 hours, to a concentration which will render milk strongly rancid within one-half hour after cooling (spontaneous milk). It is acknowledged, however, that factors other than enzyme concentration, such as the presence of a natural inhibitor of membrane lipase, may be involved in determining the degree of spontaneous lipolysis in various milks.[328]

The so-called plasma lipase is assumed not to be adsorbed onto the fat globules and is supposedly not implicated to any great extent in spontaneous rancidity. In order to facilitate lipolysis by this enzyme certain "activation treatments" such as homogenization, shaking, and additional thermal manipulations are required (see below).[328]

The plasma lipase can be almost completely removed from skim-

milk with the caseinate fraction by high speed or prolonged centrifugation or by treatment with rennet.[328] However, differential centrifugation has shown that the enzyme is not associated preferentially with any particular size of casein aggregate.[328] Skean and Overcast[306] found that casein separated by centrifugation at 50,000 rpm possessed almost twice the lipase activity of the casein separated by acidification with lactic acid. Moreover, they demonstrated that only the α-casein fraction possessed lipolytic activity. It also has been shown that separator slime is an excellent source of lipase activity. Therefore some of the lipase activity is associated with the heavier, insoluble particles normally present in milk.[232,262]

The suggestion has been advanced that casein *per se* may possess lipase activity.[252] This was based on the observation that casein acts as a strongly basic catalyst despite the fact that the pH of milk is around neutrality.[249,250] Lea *et al.*[210] have also noted that the surface of casein appears to provide a more alkaline environment than would be expected from the pH of a casein solution before drying.

Interesting investigations on the binding of l-ascorbic acid by casein led to the observation that the resultant complex had simple esterase activity. Fujimura and Hamaguchi[94,95,96,97] added ascorbic acid in varying concentrations to milk, then isolated the ascorbic acid-caseinate complex by centrifugation or alcohol precipitation. Their preparations were capable of hydrolyzing ethyl acetate to a degree proportional to the amount of bound ascorbic acid. The esterase activity of the complex gradually declined during storage of the complex, but could be practically completely restored by the addition of an optimal amount of ascorbic acid.

Unfortunately the experiments of these investigators were not extended to include other simple esters or triglycerides. However, they are intriguing enough to warrant further research on the subject of whether casein *per se* has inherent ability, alone or in combination with ascorbic acid, to catalyze the hydrolysis of triglycerides and simple esters.

It should be mentioned here that a number of studies have been conducted on the distribution of lipase activity in various fractions of milk. However, much of the early work should be interpreted with caution, because it is difficult to ascertain whether these investigations were dealing with the same, different, or one or more lipases. Also the conditions, i.e., temperature, centrifugal force, pH, size and number of fat globules, age and history of the milk undoubtedly affect the concentration and distribution of lipase activity in various fractions. These unknowingly were not standardized and the natu-

ral consequence is manifested in the many divergent reports in the literature.

Investigators have found greater activity in the skimmilk portion of milk than in the cream[152,169,219,260,262] but greater activity has also been found in the cream, rather than in skimmilk.[177]

Dorner and Widmer[55] could detect no lipase activity in whey, whereas Gould[102] has used rennet whey as a source of lipase activity. According to Palmer and Hankinson[244] the lipolytic agent in milk was largely, if not entirely removed by passing raw rennet whey or raw acid serum through a Berkefeld filter. Tarassuk and Frankel[328] and others,[2] on the other hand, found rennet whey to be practically devoid of lipase activity.

Rao[275] obtained precipitates following prolonged dialysis of raw skimmilk that showed considerable lipolytic activity. He suggested that one of the milk lipases is a globulin or is globulin-like in nature. Sumtsov[320] found that milk lipase activity was associated with the immune globulin fraction. These observations are consistent with the view that pancreatic lipase is a globulin or essentially associated with a globulin.[100]

ACTIVATION OF THE MILK LIPASE SYSTEM

In the preceding sections mention was made of the inactiveness of the milk lipase system in freshly drawn milk. Whereas a relatively small percentage of milk exhibits spontaneity, the bulk of the milk produced does not show increases in lipolysis merely by cooling and aging. Such milk must undergo certain "activation treatments" to expedite lipolysis. In this respect the lipase system in nonspontaneous milk is certainly different, for example, from the lipases found in the pancreas, which will cause relatively rapid hydrolysis of milkfat even though the milk is in the "native" state.[103] Activation treatments which have been found to accelerate lipolysis in normal milk are homogenization and various other forms of agitation such as shaking and churning, proper thermal manipulation of the milk, and the addition of chemicals.

Homogenization and Agitation

In 1932, Dorner and Widmer[54,55] ascertained that the homogenization of raw milk containing fat in the liquid condition resulted in exceedingly rapid lipolysis. It was also established at that time that the degree or intensity of the resulting rancid flavor was, within limits, related to the homogenization pressure employed. Raw milk homogenized at temperatures between 37.7° and 54.4°C. will become

rancid within a very short time, in some cases in only a few minutes.[132,354] Nilsson and Willart[234] studying the influence of homogenization on lipolysis found that homogenization although rendering milk rancid quickly, produced no noticeable difference in the rate of fatty acid liberation in milk homogenized for 30 sec., and in milk homogenized for 90 sec. if activity was determined within a few hours following homogenization. However, if the assays were conducted 24 hours following homogenization 50% more activity was apparent in the milk homogenized for 90 sec. These investigators also established that when the amount of substrate available for lipase action is not limiting, then a plateau is reached at which further fat-splitting does not occur. Although this would appear to be due to inhibition of the enzymes by the hydrolytic end-products, rehomogenization of the milk produced further activity indicating that other factors are involved.

The shaking of raw whole milk containing liquid fat also enhances lipolysis[42,49,53,68,201,293,305] as will the churning of cold milk or cream, or pumping.[363] It has been generally regarded that homogenization and most forms of mechanical agitation are essentially alike in that they accomplish activation by increasing the surface of the substrate available to the enzymes.[142,201,355] This explanation is not entirely satisfactory, for churning, unlike homogenization and shaking warm milk, reduces rather than increases the surface area of the substrate.[132] In addition, agitation by air in a vertical pipe produces much more activation than agitation in an air-free Waring Blendor.[133] It is also clear that the effectiveness of agitation is not determined by its violence alone.[222]

An explanation, at least in part, for the activating influence of agitation processes upon lipolysis has recently been suggested from a study of the occurrence of rancidity in pipeline milkers.[327] The increased use of pipeline milkers and farm tanks on dairy farms has been accompanied by an increased incidence of rancidity in market milk.[133,160,175,214,239,314,343] The trouble has been traced to "risers" in the pipelines, that is, vertical sections connecting one pipeline to another at a higher level. Air leaking into the milk lines causes considerable foaming of the warm milk lifted in the "risers" under reduced pressure. The formation of foam due to air agitation was found to be an important feature of the mechanism involved in activation of lipolysis and the resulting development of rancid milk in pipeline milkers.[327] Optimal conditions for activation by air agitation appear to be foaming with the continuous mixing of foam and milk at temperatures that keep the milkfat liquid.[327] These conditions are ap-

parently ideally provided in pipeline milkers if risers, air leaks and certain types of filters are present in the system.[327]

According to Tarassuk and Frankel[327] foam promotes lipolysis by providing (a) greatly increased surface, (b) selective concentration of enzyme at the air-liquid interface, (c) "activation" of the substrate by surface denaturation of the membrane materials around the fat globules, and (d) intimate contact of the enzyme(s), and the "activated" substrate.

The formation of foam during shaking and its relationship to lipolysis had been noted before, but no explanation regarding the role played by foam was elicited.[132] It is reasonably fair to assume that a parallelism exists between air agitation (such as that which takes place in pipeline milkers) and shaking. In the case of shaking and churning, foam formation can readily take place as well as the disruption of the natural membrane material.[86,262] However, whereas shaking may also result in some shearing of fat globules thereby increasing total surface area of substrate, this cannot be the case in churning.

Foaming would not be expected to be instrumental in the increased lipolysis due to homogenization. This was clearly illustrated by the experiments of Nilsson and Willart.[234] Also the addition of an antifoaming agent prior to homogenization does not alter the degree of activation.[328] Undoubtedly the main factors involved in activation by homogenization are the increase in the surface area of the substrate and changes in the natural adsorption layer around the newly formed fat globules. Brunner *et al.*[24–26] have reported that the proteins in the fat globule membrane in homogenized and nonhomogenized milk are different both in number and nature. It has also been shown that "resurfacing" fat globules by emulsification into skimmilk increases lipolysis.[201]

Thermal Manipulation

Unlike spontaneous milk, normal (nonspontaneous) milk requires additional thermal treatments beyond the first cooling to activate the milk lipase system. Krukovsky and Herrington[197] were the first to demonstrate that lipolysis in normal milk could be hastened by warming cold milk to 29.4°C. then recooling beyond the solidifying point of the fat. Most samples of milk subjected to this treatment will become rancid within 24 hours.[132] The temperature of 30°C. is critical and heating below or appreciably beyond that point diminishes the degree of activation which can be obtained. This type of activation is also of practical importance because it can happen acci-

dentally.[356] For example, if warm morning milk is added to a can of milk refrigerated from the night before and the whole cooled again, the milk may be rancid by the time it is ready for processing.

The fundamentals involved in inducing lipolysis by temperature fluctuations are probably quite different from those involved in activation by homogenization and shaking.[132] This is manifested by the fact that lipolysis in milk activated by cooling, warming, and cooling proceeds faster in the cold. Lipolysis in homogenized or agitated milk proceeds best at temperatures normally employed for carrying out enzymatic reactions, i.e., about 37.7°C. Spontaneous lipolysis in milk, however, is seemingly unaffected by the temperature of incubation once the milk has been cooled to the appropriate temperature.[328] In this connection, Johnson and VonGunten[163] noted that rapid cooling of herd milk gives higher free fatty acid values than does slow cooling.

Milk containing fat globules with a natural fat surface can be activated, deactivated, and reactivated by changes in temperature.[168,169] This type of activation is reversible to a certain degree. Activation by homogenization is not reversible. The phenomenon of temperature activation is found only when the fat globules have their natural layer of adsorbed material. Neither homogenized milk, or emulsions of tributyrin, nor of butteroil emulsified in skim-milk can be activated by temperature changes.[169]

Several hypotheses have been advanced to explain the peculiar effect of cooling, warming, and cooling. Krukovsky and Sharp[202] suggested that the acceleration of lipolysis by temperature changes is dependent upon the degree of solidification of the fat and upon conditions at the fat-serum interface. According to Tarassuk and Richardson,[335] the increase in lipolysis induced by temperature fluctuations is related to the permeability of the fat globule membrane to the enzymes.

Rao[275] proposed a hypothesis based on the classical Langmuir-Harkins theory of orientation in order to elucidate the underlying mechanism of temperature activation. According to this investigator, the lower and unsaturated fatty acids, being more polar and lower melting, would tend to orient themselves preferentially toward the aqueous phase as the fat is liquified. This would imply that the milk lipase system is more specific for the polar acids.

Chemical Addition

Kelly[176,178] reported that the hormone pitocin will activate the milk lipase system, but its effect seemed to vary with the substrate em-

ployed. Dunkley and Smith[60] state that calcium chloride in small amounts will accelerate lipolysis.

The milk lipase system is apparently activated by mercuric chloride. Raw milk preserved with corrosive sublimate contains, in some instances, a much larger concentration of free fatty acids than unpreserved samples. Pasteurized milk preserved in similar fashion does not show this increase in free fatty acids.[215]

Castell[32] claims that ammonia in small concentrations will increase lipolysis, but this may be a pH effect. In this regard, Ito and his co-workers[150] concluded originally that there were no enzymes in cow's milk capable of hydrolyzing tributyrin, olive oil, or methyl butyrate at pH 7.0. Later, however, they stated that these enzymes exist in milk but in an inactive state and can be activated by dilute ammonia.[149] A study of their data showed that the ammonia experiments were conducted at pH 8.0. Activity on tributyrin at pH 7.0 would not be expected in any case since this substrate is not hydrolyzed at all over short incubation periods at this pH.[232,260]

Methyl butyrate at most shows only slight hydrolysis at pH 7.0.[90]

Surface active agents such as sodium heptadecyl sulfate was reported by Packard and Jezeski[240] to slightly stimulate lipase action in normal milk both at 5°C. and at 37°C. Induced lipolysis was either unaffected or slightly accelerated by low concentrations but inhibited by high concentrations.

According to Forster *et al.*,[80] iodoacetate in concentrations of 0.01*M* and 0.001*M* activate esterases when the sodium salt of the butyryl ester of 2-naphthol-6-sulfonate is used as substrate. This is in marked contrast to the adverse effect that N-ethyl maleimide has on lipolysis.[339]

INHIBITION AND INACTIVATION OF THE MILK LIPASE SYSTEM

Thermal Inhibition

The effect of heat on the inhibition and complete inactivation of the milk lipase system has been extensively investigated. Palmer[243] found that lipolysis could be effectively retarded, if not entirely prevented, by heating fresh milk to 75°C. for a few minutes. Nair[231] could not detect lipase activity in powdered whole or skimmilk desiccated by a drying system in which the fluid milk had undergone a preliminary pasteurization at 63°–64.5°C. for 30 min.

Dorner and Widmer[55] found that homogenized milk held at 55°C. for 20 min. would not undergo lipolysis upon subsequent incubation.

However, these investigators also noted that a homogenized milk sample heated to 70°C. for 5 min. immediately following homogenization became strongly rancid in 24 hr. The rancid flavor, however, was atypical, being more of an "aromatic" rancidity compared to the sharp and bitter taste of unheated homogenized milk.

Sandelin[291] found that the lipase system in cream can be thoroughly inactivated by momentary heating at 80°C. or 30 min. exposure at 62.3°C.

Krukovsky and Herrington[198] incubated fresh, raw cream at 43.3°, 51.6°, 60.0°, 68.3°, 76.6°, and 82.2°C. for periods of time ranging from 0 to 150 min. At 43.3°C. for short incubation periods, lipolysis was first increased but then decreased upon prolonged incubation, one-third the activity being lost after 150 min. At 51.6°C., one-half the maximal activity took place at 20 min. of incubation. Lipolysis at 60°C. could still be detected after 15 min. of incubation, but not after 135 min.

Hetrick and Tracy[137] have studied the holding times requisite for the destruction of the milk lipase system at temperatures from 62.8° to 85°C. in homogenized milk. A semi-log relationship of temperature against time was observed. At 62.8°C., approximately one-third the time was required to inactivate the lipase system as was required to inactivate the enzyme phosphatase, but at 85°C. the same exposure time was sufficient to retard the activity of both enzyme systems. The findings of Mattick and Kay,[219] however, are not in accord with the above, since in their work they found the milk lipase system to be more thermolabile than the phosphatase.

The time required to inactivate the lipase system at any temperature was stated by Hetrick and Tracy[137] to vary with the rate of heating to, and cooling from, the holding temperature. Milk heated at the rate of 2.7°C. per min. required only instantaneous exposure at 61.1°C. for the complete inactivation of the lipase system, whereas instantaneous heating of the milk (within 5 sec.) with the Mallory unit required a temperature of 85°C. with instantaneous exposure. Frankel and Tarassuk[92] reported that heat-inactivation of milk lipases follows first-order kinetics.

Schwartz[296] investigated the effect of various heat treatments on the pH-activity curve of the lipase system contained in raw, lyophilized skimmilk. For this purpose the powder was reconstituted and exposed to the temperatures and times listed in Table 42. The milk was cooled to below 30°C. within 5 sec. after heating and then incubated with the substrate (cream) following adjustment of the pH to the desired level.

TABLE 42

EFFECT OF VARIOUS HEATING PROCEDURES ON LIPOLYSIS[a]

Temp., °C.	Time Sec.[b]	pH Levels						
		6.2	6.6	7.0	7.5	7.9	8.5	9.5
		Per Cent Inactivation						
60	17.4	73.5	67.4	55.0	45.8	49.7	41.3	53.5
66.8	14.2	65.1	61.4	65.7	65.4	47.8	48.4	90.7
72	14.4	91.0	90.1	84.4	83.5	82.1	83.8	91.3

[a] Data of Schwartz.[296]
[b] Milk attained temperature at or within these times.

The data of Nilsson and Willart[234] indicate that it cannot be assumed that lipases are completely inactivated by a given set of heating conditions when the heated milk is subsequently assayed for lipase activity using short incubation conditions. They report that milk heated at 80°C. for 20 sec. will show no lipase activity after subsequent incubation for 48 hr., but at 73°C. for 20 sec. the milk will be rancid after 48 hr., even though activity cannot be detected if the milk is assayed immediately after heating. Part of the data of Nilsson and Willart is reproduced in Table 43.

TABLE 43

INFLUENCE OF HEATING TIME ON THE HEAT-INACTIVATION OF MILK LIPASE(S)[a]

Temperature °C.	Heating Time, Sec.	% Inactivation Determined after Incubation for	
		3 Hr.	24 Hr.
65	5	23	20
	22	52	50
	36	70	57
	74	100	81
72	5	85	30
	22	96	91
	36	100	96
	72	100	100

[a] Data of Nilsson and Willart.[234]

Heating milk at temperatures above 75°C. for a few seconds generally eliminated lipolysis. It was noted, however, in several instances, that after an incubation period of 48 hr. appreciable activity could be detected in samples heated to 90°C. or more. This behavior closely resembles the well known reactivation phenomenon of milk phosphatase and peroxidase, but in the case of the lipases reactivation, if that is the phenomenon, was only noted in some instances.

Fat apparently protects the lipases to some extent from heat inactivation, 1° or 2°C. higher temperatures being needed for whole

milk than for the corresponding skimmilk.[92,234] The influence of fat content of milk on heat inactivation of the milk lipase system is given below:

INFLUENCE OF FAT CONTENT ON THE HEAT-INACTIVATION OF MILK LIPASE[a]

Fat, %	% Inactivation after Heating at 55°C. for 5 Min.	15 Min.
0.1	50	70
5	40	63
10	35	60
20	29	57

[a] Data of Nilsson and Willart.[234]

Greenbank and Wright[114] have indicated that the minimal temperature and time required to completely inactivate the lipase system in milk is not known. They found that certain samples of dried milk prepared from milks that had been heated for 30 min. at 61.1°, 66.6°, or 72.2°C. and stored at 30°C. developed rancidity within 112, 126, and 140 days, respectively.

Bullock[27] has declared that many delicate enzyme systems can be spray dried without loss of activity even when the entering air current is 120°C. This thermostability he claims, arises from the fact that the droplets of liquid in the drier are dried rapidly with consequent local cooling.

Photo Inhibition

The milk lipase system shows an unusual and remarkable sensitivity to light. Kay[168] exposed fresh milk in glass vessels to bright summer sunshine for 10 min. and found that 40% of the lipolytic activity was destroyed. Exposure for 30 min. resulted in a loss of 80% activity and exposure to an 800 watt (200 volt) quartz mercury-vapor lamp at a distance of 15 cm destroyed three-fourths of the lipolytic power of the milk. He noted, however, that if oxygen was first removed from the system before exposure to sunlight, the effect of the light was greatly diminished. Kannan and Basu[167] observed that in some cases exposure to ultraviolet light destroyed the lipase system and diffused daylight brought about a partial inactivation.

Frankel and Tarassuk[92] exposed a layer of raw skimmilk 1 cm thick to direct sunlight at room temperature and noted a loss in lipase activity of 84% in 5 min. and 96% after 10 min. In diffuse daylight inactivation was less, but 71% was lost in 1 hr. Loss of activity by light was independent of temperature of the milk, equal losses being observed at 0°C. and at 37°C. The enzymes were markedly protected against light-inactivation by the presence of fat.

Stadhouders and Mulder[316] confirmed Kay's[168] observation that the shorter wavelengths (about 4300 Å) of the spectrum are most destructive to milk lipase. The destructive effect of light could be repressed by reducing agents such as metol, hydroquinone, and especially by hydrogen sulfide. Ascorbic acid and methionine had no effect, but cysteine afforded a significant protection. Lipase which had been inactivated by light was not reactivated by treating the milk with hydrogen sulfide.

Chemical Inhibition

Heavy metals are known usually to affect enzymes adversely. Aside from the apparent activation of the milk lipase system by mercuric chloride, it has been reported that the addition of metals to nonhomogenized milk depresses or retards completely lipase activity. Davies[50] found that 10 ppm added copper caused complete inactivation of the lipolytic enzymes, whereas 2 ppm reduced activity some 70%. Cobalt and nickel were more powerful inhibitors than iron, chromium, or manganese, and silver (50 ppm) reduced activity approximately 30%. Frankel and Tarassuk[92] reported that raw skimmilk treated with 5–20 ppm. Cu^{++} for 15 min. at room temperature inhibited lipase activity by 7–17%, whereas 5 ppm. at 37°C. for 1 hour resulted in 69% loss of activity. There was less inhibition in the presence of fat.

Krukovsky and Sharp,[203] however, showed that copper was incapable of inhibiting lipase activity in nonhomogenized milk in the absence of oxygen. At the same time they also found that oxygen alone was an active inhibitor, its effect being greatly enhanced by the presence of small amounts of copper.

Hetrick and Tracy[137] were unable to demonstrate any drastic effects of copper on the time-temperature relationship for the inactivation of the lipase system in nonhomogenized milk.

Oddly enough, the addition of copper to homogenized milk in concentrations as high as 10 ppm is without effect on subsequent lipolysis.[102] No explanation has been forthcoming for the anomalous results noted in homogenized and nonhomogenized milk as far as the action of copper is concerned. However, several possibilities suggest themselves in way of clarification. Herald *et al.*[131] have analyzed the ash of the fat globule membrane material for constituent metals. Their results indicate that the natural membrane material contains considerable copper. A special affinity may exist, therefore, between copper and the membrane material. Copper added to nonhomogenized milk may, as a consequence, tend to be adsorbed

onto the natural membrane. Since this would also be the eventual natural environment for the lipases, inactivation may take place through whatever mechanism is involved (e.g., oxidation, binding, or chelation with the active sites of the lipase molecule) before the enzyme has an opportunity to "penetrate" the membrane material. From what has been said thus far, it is reasonable to assume that the natural membrane material offers at least transient protection of the fat against lipolysis. In homogenized milk, on the other hand, the natural membrane is probably largely removed as has been indicated by the investigations of Brunner *et al.*[24-26] Lipolysis proceeds rapidly due to the increase in available surface coupled with the elimination of the natural membrane material. Consequently, added copper may be attracted to the "sloughed" off natural membrane material, wherever it may be, and thereby removed before it reaches the sphere of action.

The differences noted in copper inactivation in homogenized and nonhomogenized milk, may be analogous, at least to some extent, to the inhibitory effect of formaldehyde in which a similar situation exists (see below).

A number of salts inhibit lipolysis in milk, the most effective being sodium chloride.[31,53,99,102,262,365] Lipolysis was found to be insignificant in cream containing four per cent sodium chloride[99] and in homogenized milk in the presence of 5–8% of this salt.[102]

Calcium chloride, sodium chloride, and the sodium, potassium, and magnesium salts of lactic acid inhibited lipolysis on tributyrin at pH 5.4 and 6.3 when present in a concentration of 0.1*M*. However, magnesium chloride and calcium lactate at the same concentration were reported to be ineffective inhibitors under the same conditions.[2] Rennet precipitation of casein was less destructive to the lipases than was precipitation by sodium chloride or lactic acid.[53]

Peterson and his associates[260] revealed that 0.6*M* phosphate buffer slightly inhibited lipolysis, but the same concentration of borate and barbiturate buffers were without effect. They also demonstrated that zinc chloride, potassium cyanide, manganese sulfate, cysteine, and magnesium chloride retarded milk lipase action to various degrees. All of these compounds were tested at pH 8.5 with tributyrin as substrate over a 30-min. incubation period. The fact that they obtained inhibition with magnesium chloride at a tenth of the concentration reported in the above study where no inhibition was encountered emphasizes that the conditions under which the inhibitor is studied are extremely important. Undoubtedly factors such as pH, time of addition of inhibitor, sequence of addition of enzyme

preparation, substrate, buffer, and inhibitor will determine, to some extent, the degree of inhibition produced. This should be especially true in milk, where a number of lipolytic enzymes are present. The effect produced under one set of conditions may not necessarily hold under another set. This has been more often than not overlooked in investigations on the milk lipase system. It has been generally assumed, for example, that if a buffer is noninhibitory at one pH value that it will also not inhibit at another pH.

N-ethyl maleimide inhibits lipase activity in milk activated by shaking, temperature fluctuations, and homogenization,[339] 0.02*M* completely inhibiting activity. An equimolar concentration of glutathione markedly reduces inhibition by N-ethyl maleimide. This reagent can also completely inhibit lipolysis in spontaneous milk. Tarassuk and Yaguchi[339] concluded on the basis of these observations that sulfhydryl groups are essential sites of activity on milk lipases. This is supported by the fact that Frankel and Tarassuk[92] showed that glutathione, hydroquinone, and potassium thiocyanate markedly increases the storage stability of milk lipase. Glutathione was the most effective of the three reducing agents.

Chandan and Shahani[33] have reported that aureomycin, penicillin, streptomycin, and terramycin reduce lipase activity in milk by 7–49%. They found a direct relationship between concentration and inhibition with antibiotic concentrations up to 10 ppm.

Trypsin-treated raw skimmilk shows reduced lipolytic activity.[92] Milk treated with 5 to 20 ppm of the enzyme showed diminished lipolytic activity from 47 to 100%. Trypsin inactivation was not effected by the presence of milkfat. Pepsin failed to inhibit lipolysis under the same conditions.

Hydrogen peroxide in concentrations ranging from 1 to 10 ppm was found to inhibit lipase action from 31 to 92% when added to raw skimmilk for 15 min. at room temperature. Less loss occurred in the presence of fat.

The most studied chemical inhibitor of lipolysis in milk has been formaldehyde. This arose from the fact that formaldehyde had been widely used as a preservative in milk lipase studies without knowledge of its effect. For example, Palmer[241] used formalin solution as a preservative in experiments designed specifically to elucidate whether or not a lipase was normal to milk. He assumed that this reagent in concentrations ranging from 1:1500 to 1:2000 would have no detrimental effect on milk lipase if present, since these concentrations of formalin had not inhibited steapsin. Palmer could

detect no lipolysis in the presence of formalin and concluded originally that milk was devoid of a natural lipase.[242]

The effect of formaldehyde has since been studied with the knowledge at hand that a lipase system is inherent to milk. Herrington and Krukovsky[135] added from 1 to 27 drops of formalin to 160 ml of nonhomogenized 20% cream, and found that, in all cases, the lipase activity was reduced about 70%. Similarly, Roahen and Sommer[282] noted that 0.5 ml of formalin was as effective as 1.5 ml in inhibiting lipolytic activity in 100 gm. of nonhomogenized milk. Peterson *et al.*[260] reported 75% inhibition at pH 8.5 when the substrate was tributyrin and the formaldehyde was present in a concentration of 0.2%. However, this same concentration of inhibitor resulted in only 50% inhibition at pH 5.0 to 6.6.[2] Formaldehyde also inhibits at least one of the esterases in milk.[81,91]

Not all investigators have found formalin to be inhibitory. Tarassuk reported a study of the milk from one cow in which formalin did not influence lipase activity.[325] According to Tarassuk and Richardson,[335] the addition of formalin to a sample of nonhomogenized milk actually activated the lipase system. Gould[102] found formalin to be ineffective as a lipase inhibitor when added to homogenized milk in considerable concentration.

An extensive study of the effects of formalin in milk lipolysis inhibition has been published by Schwartz *et al.*[299] They found that formaldehyde acts as a competitive inhibitor and also, under the proper conditions, selectively inhibits the lipases of raw skimmilk. Frankel and Tarassuk[91] have also presented data which tend to substantiate the proposal that formaldehyde acts competitively with the substrate for the enzymes.

Schwartz *et al.*[299] showed that the inhibitory effect of formaldehyde was dependent on such factors as pH, time of addition of the inhibitor, length of the incubation period, concentration and availability of the substrate, and concentration of the inhibitor. Many of the conflicting results encountered with formalin as mentioned above, can be explained on the basis of dependence on one or more of these factors.

GENERAL PROPERTIES OF THE MILK LIPASE SYSTEM

The activity of the milk lipase system is determined to a large extent by a number of environmental factors, a knowledge of which is important to the understanding and control of rancidity. Some of the more pertinent of these are discussed below. It should be borne in mind, however, that a substantial amount of work in this regard was carried out on the assumption that only one lipase was normal to

milk. The recent evidence tending to demonstrate the presence in milk of multiple lipolytic enzymes, as well as at least one esterase proper, devaluates some of the older data, and especially their original interpretations.

Specificity

The majority of the lipases found in nature exhibit rather low specificity. That is, virtually any triglyceride will be split to some degree, regardless of the length of the fatty acid chains, whether they are saturated or not, and whether all hydroxyl groups of the glycerol residue are esterified or not. It is, at the present time, practically impossible to obtain data on the absolute specificity of lipases, because of the limitations imposed by reaction in a two-phase system. In fact, the gathering of data on relative specificities must necessarily be obtained on the supposition that the various emulsions of triglycerides under investigation all offer the same total surface area for lipase action.[295]

The milk lipase system has been reported to attack, besides milkfat, the following oils and fats: triacetin, tributyrin, tricaproin,[180] coconut, castor, cottonseed, corn, linseed and olive oils, margarine, lard and a hydrogenated fat.[103] In addition, diacetin,[179,231] diglycol laurate and diglycol oleate[244] have been reportedly cleaved. The splitting of simple esters such as ethyl acetate,[94,95] ethyl butyrate[287,359] alpha-naphthyl esters,[80,111] Tween 20,[89–91,328] methyl butyrate,[89–91,328] ethyl myristate,[244] butyl stearate,[244] N,N′-diisopropylphosphorodiamino fluoridate, tetraethylpyrophosphate, diisopropylfluorophosphate, and diethyl-p-nitro-phenylthiophosphate[78] has also been shown. However, by definition it must be assumed at this time, that these were hydrolyzed by milk esterase(s) proper.

Although the milk lipase system will attack the oils, fats and diglycerides mentioned above, nothing is known concerning the relative rates of their hydrolysis. From a practical standpoint, only the natural substrate (milkfat) is of importance. To undertake a study of the relative affinity of the milk lipase system toward the fatty acids which make up milkfat would be a formidable task. Since there is random distribution of the fatty acids within the glycerides which make up milkfat,[313] the position of the individual fatty acids in the glycerides may affect specificity and undoubtedly influences the rate of cleavage of a given fatty acid. However, attempts in this direction are being undertaken, aided by the powerful analytical tool, gas chromatography. Jensen *et al.*[155] have determined that the milk lipase system is specific for the fatty acids in the alpha posi-

tion. Diglycerides formed from milk lipase action have been analyzed by Jensen *et al.*[157] for fatty acids. The pattern found was distinctly different than that of the intact triglycerides. Patton *et al.*[255] studied the action of pancreatic lipase on milkfat and found that the beta-monoglycerides produced were somewhat enriched in the C_{10} to C_{16} and especially the C_{14} saturated acids. They were poorer in the unsaturated acids.

Harwalkar and Calbert[129] reported that lauric and higher acids are the major ones released at all stages of lipolysis, but as lipolysis progressed the ratio of these acids to butyric decreased. Very little change was observed in the mole percentages of the other acid fractions.

Jensen and Gander[156] established that the concentration of butyric, stearic and oleic acids in the monoglyceride fraction of lipolyzed milkfat was much lower than in intact normal milk. Myristic acid was found to be higher in lipolyzed milkfat than it is in milkfat isolated from milk not subjected to lipase action.

pH

Enzymes exert their catalytic influence usually over a somewhat restricted pH range. Within this range the activity passes through a maximum, commonly called the optimum pH, and then falls off again. Although the pH optimum is generally characteristic of a given enzyme, it may, in some cases, be altered by such things as type and strength of buffer, ionic strength, temperature, and type of substrate employed. Many of these variables have been overlooked in some instances, in establishing the pH range, and pH optimum for the activity of the milk lipase system.

Bosco[22] found that lipase action on milkfat ceases at pH of about 4.7, whereas, Schwartz *et al.*[298] reported no activity at pH 5.2. The lipase system in milk is more active on milkfat at pH levels between 6.0 and 7.5 than it is on tributyrin in this range.[60,232,296] The lipases and esterase(s) in milk are sensitive to extremes in pH and even in the vicinity of the pH optimum, where enzymes are supposedly more stable to heat and to inactivation by acids and alkalis, marked inhibition may occur.[91] Incubating raw skimmilk at pH 6.0 and at pH 8.9 for 1 hour at 37°C. prior to assay has shown that 47 and 40% inactivation occurred when milkfat was employed as substrate, and 67% inactivation took place at both pH levels when tributyrin was the substrate.[91] Even more inactivation was noted when simple esters were assayed. Although some inactivation may have been

due to temperature inactivation, most of it is probably attributable to pH exposure.

The temperature of incubation of skimmilk with tributyrin markedly influences the points where activity is no longer perceptible, but no similar data on milkfat is available.[60] This is unfortunate since the possibility exists that lipolytic action due to the natural enzymes in milk may take place in certain types of raw milk cheeses.

It has been reported that there are lipases and esterases in milk operating in the pH range 5.0 to 6.6 and able to hydrolyze tributyrin and simple esters.[2] The lipases were reported to have pH optima at 5.4 and 6.3 and were claimed to be inhibited by formaldehyde. These lipases would, if the data are valid, have the peculiar property of operating over a pH range of only 0.5 to approximately one pH unit.

Such a narrow pH range of activity is unusual indeed and may indicate that nonenzymatic entities are present in milk which can promote hydrolysis of triglycerides and simple esters (*cf.* section on nature). Prior to these observations, no activity on tributyrin had been detected at pH levels approximating that of normal milk. These discrepancies were explained on the basis of the acid used for adjusting the acidity of the medium. Acids such as sulfuric, hydrochloric, phosphoric, and citric were claimed to be inhibitory relative to lactic acid.[2]

Apparent Temperature Optimum

A rise in temperature has a dual effect upon an enzyme-catalyzed reaction: It increases the rate of the reaction but it also increases the rate of thermal inactivation of the enzyme itself. Like the pH optimum, the temperature optimum may, in some instances, be altered by environmental conditions, e.g., pH, type and strength of buffer, etc. The term "temperature optimum," therefore, is useless unless the incubation time and conditions are specified. A more enlightening nomenclature is "apparent temperature optimum" which indicates that the optimum has been obtained under a certain set of conditions, and may or may not hold when conditions are changed.

The apparent temperature optimum for the milk lipase system is reported to be around 37°C. both on milkfat and on tributyrin.[89,282,301,312] This temperature has been recorded both at pH 8.9 and pH 6.6 for milkfat,[89,282,301] and at pH 8.0 and pH 6.6 on tributyrin.[89,312]

Stadhouders and Mulder,[315] however, using tributyrin and milkfat together as substrates in milk at pH 9.1 found that, under their condi-

tions, poorer activity was apparent at 37° relative to 25° or 15°C. after 1, 4, and 24 hr. of incubation. In fact, 90% less activity occurred at 37° at 24 hr. of incubation than at 15°C. The activity at 15°C. was quite constant, indicating complete stability of the lipase system under the conditions used.

It seems unlikely that the so-called membrane lipase, i.e., that enzyme primarily implicated in rancidity in spontaneous milk, would show an optimum at 37°C. The older work in this connection cannot be evaluated with certainty since the type of milk involved and its subsequent handling are not absolutely defined. The data of Tarassuk and Frankel,[328] however, show that the cooling of spontaneous milk followed by aging at 5° and 35°C., respectively, results in only slightly more activity in milk aged at the higher temperature. It is also reported that incubating precooled spontaneous milk at 37°C. may not necessarily increase lipolysis over the same milk aged in the cold.[328] Eventual data on the temperature-activity curve of the membrane lipase may, therefore, reveal some interesting and probably unique characteristics of this enzyme.

Stability

It has been noted from time to time that some loss of lipase and esterase activity takes place when raw milk is stored in the fluid state prior to assay. Peterson *et al.*[260] held fresh whole milk at 0°C. for zero to 15 hours, subsequently skimmed the milk and incubated the skim portion with tributyrin at pH 8.5. Of four milks each from a different cow subjected to this treatment, one showed no loss in activity, another only slight loss, and the other two definite decreases in activity. They interpreted these observations as indicative that some milks contain at least two lipases, one more stable than the other. In the light of the contributions of Tarassuk and his school, this may not necessarily be the only explanation, since the membrane lipase which is purported to be present in all raw milk in various concentrations, would be expected to adsorb onto the fat globule membrane material. This then could account for the variable loss in activity in their samples. However, Tarassuk and Frankel[327] have reported that the storage of milk for 24 hr. at 0°–4°C. before activation by homogenization or agitation decreases the extent of lipolysis 20 to 50% below that of the same milk activated without aging. In the case of "temperature activation" the lipolytic activity decreased 80 to 90% after the same length of storage in the cold. Other experimental details were not presented, and it is not clear, therefore, whether the same sample of milk was used in all experiments. If

this was the case, then different lipases are certainly involved in lipolysis induced by temperature activation, and that induced by homogenization and/or shaking, provided that the assumption is made that "aging" has no effect on the condition of the substrate.

Schwartz[296] found that raw skimmilk which had been prepared from once-cooled whole milk showed no loss in activity at pH 6.2, 6.6, 7.0, 7.5 and 8.5 after the milk had been stored at pH 6.6 for six hours in the dark at 4°C. prior to assay with the substrate (cream). In this instance, the membrane lipase would have been partially or completely absent from the skimmilk under investigation due to the prior history of the milk which he used.

The fact that Schwartz found the lipases in skimmilk to be stable under his conditions, does not of course exclude the possibility that in the interim between milking and the preparation of the enzyme source which he used, that some inactivation had occurred. Indeed some inactivation should be expected in all milk since oxygen is reported to be an active inhibitor of lipolysis.[203] In this regard, Forster *et al.*[80] advocated the deaeration of milk by passing in nitrogen at the barn as a means of preserving the original esterase activity of milk.

Frankel and Tarassuk[92] permitted raw skimmilk to stand one hour at 37°C. at pH levels ranging from 5.2 to 9.8 and found greatest activity in the range pH 6.6 to 7.6 when the milk was subsequently incubated with substrate at pH 8.9. Hence, it appears that normal milk is in an optimum pH range for stability of the milk lipase system. The same pH range of optimum stability was found in whole milk.

The stability of the lipase system in raw, lyophilized skimmilk has been investigated by Schwartz.[296] Freeze-dried raw, skimmilk powder stored in amber glass bottles at 4°C. shows no loss in lipase activity over a seven-month period when subsequently assayed at pH 6.6, 7.9, and 8.5. Here again the possibility of inactivation reaching a constant value during the time lapse of milking to preparation of the powder cannot be overlooked.

SOME EFFECTS OF LIPOLYSIS IN MILK

Besides changing the natural flavor of milk, lipolysis may produce a variety of effects, some of which may be used advantageously, whereas others may be detrimental.

One of the most noticeable changes in milk which occurs as lipolysis proceeds is a decrease in surface tension.[31,58,60,189,335–338] Fatty acids, and more especially mono- and diglycerides are good surface

tension depressants (see under methods). Milkfat obtained from milk subject to lipase action gives lower interfacial tensions with water than does milkfat obtained from milk not subjected to lipase action.[31,62]

Rancid milk and cream are difficult to churn and also inhibit the clotting of milk by rennet.[333] Krukovsky and Sharp[200] and Keith and Fouts[173] have examined the effect of lipolysis on the churnability of cream obtained from cows in late lactation. Krukovsky and Sharp[200] have concluded that the difficulty encountered in churning and the abnormal foam formation encountered in such creams is probably due largely to lipolytic action and to the concentration of the resultant fatty acid soaps at the air-plasma interface.

Tarassuk and Richardson[336] noted that the higher saturated fatty acids inhibit the milk-clotting ability of rennet, whereas the lower fatty acids enhanced clotting. The inhibitory effect of the higher fatty acids could be nullified by the addition of calcium chloride.

As little as 0.1% rancid milkfat proved to be a very effective foam depressant during the condensing of skimmilk and whey.[23] This effect was attributed to the mono- and diglycerides present.

Sager[290] has reported that rancid milkfat is assimilated by young children even more rapidly than is homogenized milkfat. This also was found to be due to the surface activity of the mono- and diglycerides.

Mukherjee[229] has repeated the observation of Greenbank and Holm[112] that fat containing free fatty acids is more susceptible to oxidation. Milkfat held under various conditions at 37°C., and examined periodically for 85 days showed increased peroxide values and color in the Kreis test in those samples having higher free fatty acid content. The possible relationship between lipase activity and oxidation in market milk deserves more attention than it has received.

An important observation made by Manus and Bendixen[215] indicates that lipolytic action takes place in composite samples preserved with corrosive sublimate (mercuric chloride). This action was shown to decrease the reading of the Babcock test. In some instances this reading was reduced over 0.15%. This loss can be attributed to the decrease in fat solubility of some of the products of lipolysis.

Koestler[187] noted an inhibitory effect of rancid milk on the growth of *Streptococcus lactis*. Koestler *et al.*[188] reported that rancid milk significantly inhibits the growth of bacteria in general, and of *S. lactis* in particular. Anderson[3,6] has stated that rancidity in milk may reach such a degree as to actually render the product sterile.

Tarrasuk and Smith[338] attributed the inhibitory effect of rancid ıilk to changes in the surface tension of the medium, but Costilow ıd Speck[40,41] believe that the inhibition is due to the toxic effect of ıe individual fatty acids.

METHODS FOR DETERMINING LIPASE ACTIVITY IN MILK

A number of methods are available for following lipase activity in ıilk. The fact that numerous modifications and variations have een introduced, attests to the limitations involved and to the un-ıtisfactory nature of all methods developed thus far.

Fundamentally, the methods available now may be grouped as)llows: (1) titration before or after the isolation of the unesterified ıtty acids; and (2) following changes in surface tension. The use of mple esters as substrate which give or can be made to give a color)llowing hydrolysis have been advocated and utilized. However,)r the sake of definition, these must be necessarily limited to the ac-ion of the esterase(s) proper, unless it can be shown otherwise.

'itration

Titration of the fatty acids produced by the action of the milk pase system on milkfat or other triglycerides has been the most ;idely used method for following activity. Basically, four varia-ions have been employed: (a) direct titration of the reaction me-ium alone or in the presence of added solvents; (b) separation of the pid phase by churning or extraction and its subsequent titration; (c) istillation followed by titration of the distillate; and (d) adsorption f part or all of the reaction medium on a support followed by elution f the fatty acids and the determination of their titer.

Direct titration of the entire milk system before and after incuba-ion was one of the earliest procedures used for following lipase activ-ty.[5,107,205,242,284,287] Addition of a fat solvent to the milk before titra-ion has also been applied.[60,260,261,275]

In an effort to increase sensitivity, methods for the removal of the ipoidal constituents present in milk were developed and used exten-ively. The churning procedure for the removal of the fat for titra-ion was widely used.[85,104,135,136,197–200,202,336] This consisted essentially)f churning the cream obtained from milk subjected to lipolytic ac-ion in order to obtain the fat, followed by clarification, centrifuga-ion, filtration and titration of the resultant butteroil. Formalin ıas been reported to interfere with this procedure.[283] Gould[104] con-:luded that the free fatty acids in the butteroil obtained by churning nethods were not responsible for the typical rancid flavor in dairy

products. It is to be expected that some difficulty is likely to be encountered in churning due to the surface activity of the products of lipolysis.

Methods for removal of the lipid material from milk by solvent extraction for the purpose of titration have also been widely employed. Various solvents have been utilized for extracting the lipid material including boiling ethanol,[101,102,104] a mixture of ethanol, ethyl ether and Skelly-solve F or other petroleum ether.[88,161,162]

The extraction-titration method of Frankel and Tarassuk[88] recovers 95–100% of the higher molecular weight acids, and 52–58% of the lower acids. Poor recovery of the lower fatty acids is encountered in all of the solvent extraction methods.

Stadhouders and Mulder[315] have proposed an extraction-titration method employing tributyrin as substrate.

Distillation

The fact that milkfat is unique in that it contains relatively appreciable amounts of steam distillable fatty acids has led many workers to utilize steam distillation as a method for following lipolysis.[63,85,167,168,180,219,282] Determination of the volatile free fatty acids usually involves solution of the fat in ether, extraction of the acids in alkali, acidification of the extract with a nonvolatile acid, steam distillation of the mixture to constant volume, and titration of the distillate. This method has distinct disadvantages when milkfat is employed as substrate, the most obvious being its inability to recover the nonsteam distillable acids.

Adsorption

Smith[308] was one of the first investigators to demonstrate that the partition chromatogram developed by Martin and Synge[217] for amino acid esters was capable of separating a mixture of fatty acids. Elsden[67] extended the method to separate acetic, propionic, n-butyric and n-valeric acids. Moyle *et al.*[228] were able to identify naturally occurring fatty acids from acetic to caprylic acids by modifying Elsden's method. Ramsey and Patterson[272–274] devised a procedure for separating acids containing 1 to 19 carbon atoms. However, their procedure necessitated preliminary fractionation of the acids in groups of certain chain length before chromatographing.

Adsorption and partition techniques on inert supports have recently been applied for the estimation of fatty acids in dairy products.[279] Achaya[1] has used Elsden's method in a study of rancid ghee. Harper[126] has developed a chromatographic technique for the separa-

tion of acetic, propionic, and butyric acids in cheese that requires no preliminary extraction of the acids from the sample.

Keeney[170] and Harper and Armstrong[127] have applied a chromatographic technique for the detection of foreign fats in dairy products. Harper *et al.*[128] suggested a silica gel technique which recovers at least 90% of added fatty acids in approximately 15 min.

Surface Tension

Efforts have been expended to apply surface tension measurements for determining the degree of lipolysis in the milk.[58,60,61,93,244,245,329,334,336–338] As mentioned earlier, the hydrolysis products resulting from lipase action in milk are strongly surface active. Tarassuk and Regan[334] have stated that the lowering of surface tension produced as a result of lipase action is the most distinct change differentiating rancid from nonrancid milk. However, a great many variables influence the surface tension of milk. Jensen and Morgan[159] pointed out that the elaboration of structurally different mono- and diglycerides and their concentration under different conditions may markedly affect the reading.

Miscellaneous

Jensen and Gander[156] have described a procedure for estimating the monoglyceride content of normal and rancid milk based on periodate oxidation of the alpha- mono-glycerides and determination of the formaldehyde liberated in the reaction. Beta-monoglycerides are isomerized to monoglycerides and determined by difference. However, analysis of rancid milk showed that neither the alpha- or beta-monoglycerides parallel the free fatty acid content.

PART II AUTOXIDATION

Owen W. Parks

Lipid autoxidation in fluid milk and a number of its products has been a concern of the dairy industry for a number of years. The need for low temperature refrigeration of butter and butteroil, and inert gas or vacuum packing of dry milks to prevent or retard lipid deterioration, in addition to the loss of fluid and condensed milks as a result of oxidative deterioration have been major problems of the industry.

The autoxidation of milk lipids is not unlike that of lipids in other edible products. However, the complex composition of dairy products, physical state of the product (liquid, solid, emulsion, etc.), presence of natural anti- or pro-oxidants, as well as processing, manufac-

turing, and storage conditions tend to influence the rate of autoxidation in addition to the composition and amount of autoxidation products formed.

The literature dealing with the autoxidative mechanism involved in lipid deterioration has been concerned with investigations on pure unsaturated fatty acids and their esters. The reactions involved, however, are representative of the reactions occurring in lipids and lipid-containing food products.

Autoxidation Mechanism

The initial step in the autoxidation of unsaturated fatty acids and their esters is the formation of free radicals. Although little is known regarding the initiation of such radicals, the resulting free radical chain reaction has been elucidated based on the investigations of Farmer and Sutton.[70] In the case of mono-unsaturated and non-conjugated polyene fatty acids, those acids of significance in milkfat, the reaction is initiated by the removal of a hydrogen atom from the methylene (α-methylene) group adjacent to the double bond (I). The resulting free radical, stabilized by resonance, adds oxygen to form peroxide-containing free radicals (II); these in turn react with another mole of unsaturated compound to produce two isomeric hydroperoxides in addition to free radicals (III) capable of continuing the chain reaction.

$$-CH_2-CH=CH-CH_2- \xrightarrow{-H} -CH\cdot-CH=CH-CH_2- \leftrightarrow -CH=CH-CH\cdot-CH_2-$$

(I)

$$-CH\cdot-CH=CH-CH_2- \xrightarrow{+O_2} -CH(OO\cdot)-CH=CH-CH_2-$$

$$-CH=CH-CH\cdot-CH_2- \xrightarrow{+O_2} -CH=CH-CH(OO\cdot)-CH_2-$$

(II)

$$-CH(OO\cdot)-CH=CH-CH_2- + -CH_2-CH=CH-CH_2- \rightarrow -CH(OOH)-CH=CH-CH_2- + -CH\cdot-CH=CH-CH_2-$$

$$-CH=CH-CH(OO\cdot)-CH_2- + -CH_2-CH=CH-CH_2- \rightarrow -CH=CH-CH(OOH)-CH_2- + -CH\cdot-CH=CH-CH_2-$$

(III)

Oleic acid, having two α-methylene groups, gives rise to four isomeric hydroperoxides which have been isolated in equal amounts by various workers.[70,268,289] The preferential points of attack in poly-ene

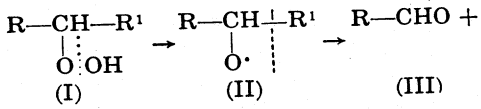
nonconjugated systems are the α-methylene groups located between the double bonds. Hence, the autoxidation of linoleic acid and linolenic acid can theoretically result in the formation of three and six isomeric hydroperoxides respectively.

However, a characteristic of hydroperoxide formation is the shifting of double bonds to form the conjugated system.[30,267] In this respect, it has been observed that almost all hydroperoxides of linoleic acid and linolenic acid are characterized by the presence of conjugated systems; the existence of an 11- linoleate hydroperoxide or an 11- or 14-linolenate hydroperoxide could not be confirmed by Badings[13] and Frankel *et al.*[86A]

In addition to the formation of hydroperoxides, other free radical reactions occur simultaneously. The formation of polyperoxides, carbon to carbon polymerization, and the formation of epoxides, and cyclic peroxides have been proposed or demonstrated in lipid oxidation—the subjects of which are not within the scope of this book.

Products of Oxidation

The hydroperoxides formed in the autoxidation of unsaturated fatty acids are unstable and readily decompose. The main products of hydroperoxide decomposition are saturated and unsaturated aldehydes. The mechanism suggested for the formation of aldehydes involves the cleavage of the isomeric hydroperoxide (I) to the alkoxyl radical (II) which undergoes carbon to carbon fission to form the aldehyde (III).[87]



Other reaction products such as ketones,[16] alcohols,[16] and semi-aldehydes[87] have been reported to result from hydroperoxide decomposition.

Table 44 includes the carbonyls resulting from the dismutation of hydroperoxides which are theoretically possible in the autoxidation of oleic, linoleic, and linolenic acids—the major unsaturated acids of butterfat. In addition to those carbonyls theoretically possible, various others have been isolated and identified as the result of the autoxidation of pure fatty acids or their esters. It has been suggested that their presence may be a result of further migration of double bonds[14] or the autoxidation of unsaturated aldehydes initially formed.[13] The characterization of nonconjugated unsaturated aldehydes is made difficult by the tendency of unsaturated com-

TABLE 44

ALDEHYDES WHICH MAY FORM FROM DISMUTATION OF HYDROPEROXIDES

Fatty Acid	Hydroperoxide[a]	Aldehydes
Oleic acid	$CH_3—(CH_2)_6—CH(OOH)—CH=CH—(CH_2)_7—COOH$	Octanal[98b]
	$CH_3—(CH_2)_6—CH=CH—CH(OOH)—(CH_2)_7—COOH$	2-decenal[98b]
	$CH_3—(CH_2)_7—CH=CH—CH(OOH)—(CH_2)_6—COOH$	2-undecenal[98b]
	$CH_3—(CH_2)_7—CH(OOH)—CH=CH—(CH_2)_6—COOH$	Nonanal[98b]
Linoleic acid	$CH_3—(CH_2)_4—CH(OOH)—CH=CH—CH=CH—(CH_2)_7—COOH$	Hexanal[13,98b]
	$CH_3—(CH_2)_4—CH=CH—CH=CH—CH(OOH)—(CH_2)_7—COOH$	2,4-decadienal[13,98,25 4b]
	$CH_3—(CH_2)_4—CH=CH—CH(OOH)—CH=CH—(CH_2)_7—COOH$[a]	2-octenal[13,98b]
Linolenic acid	$CH_3—CH_2—CH(OOH)—CH=CH—CH=CH—CH_2—CH=CH—(CH_2)_7—COOH$	Propanal[98b]
	$CH_3—CH_2—CH=CH—CH(OOH)—CH=CH—CH_2—CH=CH—(CH_2)_7—COOH$[a]	2-pentenal[98b]
	$CH_3—CH_2—CH=CH—CH=CH—CH(OOH)—CH_2—CH=CH—(CH_2)_7—COOH$	2,4-heptadienal[98b]
	$CH_3—CH_2—CH=CH—CH_2—CH(OOH)—CH=CH—CH=CH—(CH_2)_7—COOH$	3-hexenal
	$CH_3—CH_2—CH=CH—CH_2—CH=CH—CH(OOH)—CH=CH—(CH_2)_7—COOH$[a]	2,5-octadienal
	$CH_3—CH_2—CH=CH—CH_2—CH=CH—CH=CH—CH(OOH)—(CH_2)_7—COOH$	2,4,7-decatrienal

[a] Hypothetical.
[b] Reference to aldehyde identified from autoxidized fatty acid.

pounds to form conjugated systems. The identification of *cis*-3-hexenal in autoxidized soy bean oil,[144] however, lends support to the formation of such compounds in the autoxidation of unsaturated fatty acids. Thus, as a result of the complexity of the unsaturated fatty acid content of butterfat, the autoxidation of dairy products can lead to a multitude of saturated and unsaturated aldehydes.

Oxidation and Off-Flavors

The overwhelming consideration in regard to lipid deterioration is the resulting off-flavors. Aldehydes, both saturated and unsaturated impart characteristic off-flavors at minute concentrations. Terms such as painty, orange oil, nutty, shrimp-like, green leaves, tallowy, oily, cardboard, fishy, cucumber, etc., have been used to characterize the flavors imparted by individual saturated and unsaturated aldehydes as well as mixtures of these compounds.[57,74] Moreover, the small quantities necessary to impart off-flavors is such that the oxidative deterioration need not progress substantially prior to the detection of off-flavors. Patton *et al.*[254] reported that 2,4-decadienal which exhibits a deep-fried fat or oily flavor is detectable in aqueous solution at levels approaching 0.5 parts per billion. Generally speaking, the threshold values for aldehydes decrease with increasing unsaturation, increasing chain length, and vary with the medium in which they are present.[72,211] With respect to the latter point, the flavor potency of these compounds is approximately one thousand times greater in an aqueous medium than in a fat or oil. Hence, the extent of oxidative deterioration of fluid milk need not progress to the same point as that in butter, prior to the onset of off-flavors in the fluid product.

Measurement of Fat Oxidation

Various methods have been employed to measure the extent of autoxidation in lipids and lipid-containing food products. For obvious reasons, such methods should be capable of detecting the autoxidative process prior to the onset of the off-flavors. Milk and its products which develop the characteristic off-flavors at low levels of oxidation, require procedures which are extremely sensitive to oxidative changes. Thus, methods for measuring the decrease in unsaturation (Iodine No.) or increase in diene conjugation as a result of the reaction, do not lend themselves to quality control procedures.

Several methods have been introduced which express the degree of oxidative deterioration in terms of hydroperoxides per unit weight of fat. The one most commonly used has been the liberation of iodine from

potassium iodide;[3,208] wherein the amount of iodine supposedly liberated by the hydroperoxides is used as the criterion for the extent of the oxidative reaction. The colorimetric ferric thiocyanate procedure adapted to dairy products by Loftus-Hills and Thiel[141] with modifications by various workers[264,318] involves the conversion of the ferrous ion to the ferric state in the presence of ammonium thiocyanate, presumably by the hydroperoxides present, to yield the red pigment ferric thiocyanate. The latter method is reportedly[141,153] more sensitive than those methods relying on the liberation of iodine from potassium iodide. Nevertheless, any method based on the direct or indirect determination of hydroperoxides do not consider previous dismutations of these primary reaction products, and are, therefore, not necessarily truly indicative of the extent of the reaction. It is of interest to note that organoleptic detection of off-flavors in dairy products occurs at peroxide values approaching 1.0.[141,311] Higher values in other edible fats, prior to the onset of off-flavors, are not uncommon.

The Kreis test[190] based on the reaction of phloroglucinol and oxidized fats in the presence of concentrated HCl and the 2-thiobarbituric acid test[59] have also been adapted to measuring the degree of lipid deterioration in dairy products—the latter method apparently more sensitive to oxidative changes.[257] Evidence has been presented which seems to indicate that the red pigment determined spectrophotometrically in both tests is the reaction product of malonic dialdehyde with the test reagents.[257,258,303]

In addition to the previously mentioned chemical tests, methods based on the carbonyl content in oxidizing fats have also been suggested[130] as a measure of oxidative deterioration. Their value as an index of the extent of autoxidative changes is questionable, although carbonyl contents have been significantly correlated with the off-flavors themselves.[212]

Antioxidants

The use of synthetic antioxidants in the prevention or retardation of autoxidation in lipids and lipid-containing food products has been the subject of numerous investigations. Although the present U.S. standards do not permit antioxidants in dairy products, and hence the question of their effectiveness is one of theoretical interest, they are of practical interest in other countries where their use is permitted. Many compounds containing two or more phenolic hydroxy groups such as propyl gallate,[36] butylated hydroxyanisole,[350] norhydroguaiaretic acid,[204] hydroxyquinone,[109] and dihydroquercetin[277]

TABLE 45

CARBONYLS IDENTIFIED IN AUTOXIDIZED DAIRY PRODUCTS

Product	Methyl Ketone	Alkanal	Alk-2-Enal	Alk-2,4-Dienal
Phospholipids of butter[56a]	...	C_2 to C_{18}[b]	C_5 to C_{15}[b]	C_8, C_9
Butteroil[52]	C_3, C_5, C_7, C_9, C_{11}, C_{13}, C_{15}	C_1 to C_{10}	C_4 to C_{11}	C_7
Butteroil[66]	C_5, C_7	C_2 to C_9	C_5 to C_{10}	C_7, C_{10}
Fishy, butteroil[73]	C_3, C_5, C_7, C_9, C_{11}	C_3 C_5 to C_{10}	C_3 C_5, C_6, C_8, C_9	C_7
Tallowy, painty butteroil[75]	C_7	C_5 to C_{10}	C_5 to C_{10}	C_7
Butteroil[98]	C_3, C_5, C_7, C_9, C_{11}, C_{13}, C_{15}	C_1 to C_3 C_5 to C_{10}	C_4 to C_{11}	C_7, C_9 C_{10}, C_{11}
Skimmilk[66,77]	...	C_2, C_6	C_4 to C_{11}	C_6 to C_{11}
Nonfat dry-milk[15]	C_3, C_4	C_1, C_2 C_6 to C_{10} C_{12}, C_{14} Methylpropanal 3-methylbutanal	...	...
Dry whole milk[246]	C_3, C_4, C_5, C_6, C_7, C_9, C_{11}, C_{13}, C_{15}	C_1 to C_3 C_5 to C_{10} C_{12}	C_5 to C_{11}	...
Washed Cream[74]	C_7	C_5 to C_9	C_5 to C_9	C_7

[a] Reference.
[b] Includes aldehydes released from plasmalogens.

have been employed as antioxidants in studies on dairy products. These compounds apparently exert their influence by interrupting the chain reaction in autoxidation—by the capture of free radicals necessary for hydroperoxide formation.[14]

Synergists, such as the polybasic acids citric and phosphoric, have been used in conjunction with antioxidants. These compounds have no antioxygenic value in themselves, but increase the effectiveness of antioxidants. The nature of their influence may be due to the sequestering of metallic ions[14,153] or inhibiting the antioxidant catalysis of peroxide decomposition.[269] Evidently, these synergists, like the phenolic antioxidants are capable of performing a dual role of retarding autoxidation at low levels and accelerating autoxidation at higher levels.[269]

In addition to antioxidants alone or in the presence of synergists,

chelating compounds such as the various salts of ethylenediamine tetra-acetic acid[8,184] have also proven their effectiveness as inhibitors of autoxidation.

OXIDATIVE DETERIORATION IN DAIRY PRODUCTS

The literature pertaining to the oxidative deterioration of milk and its products is voluminous,[110,278] attesting to the interest in this problem. The overwhelming majority of these investigations are concerned with factors promoting or inhibiting the development of off-flavors as a direct result of the oxidative reaction. The off-flavor developing in fluid milk is commonly referred to as the oxidized flavor although such terms as cappy and cardboard have been frequently used. In contrast, the off-flavor in dry whole milk and butteroil has been described as resembling an oily or tallowy flavor, while terms such as fishy, painty, and metallic have also been used to characterize the off-flavors in dairy products.

On the basis of conflicting flavor criticisms, it has been suggested that different lipid constituents or reaction mechanisms may be involved in the oxidative process, giving rise to different flavor compounds or varying concentrations of the same compounds. In this respect, the chemical analyses of several workers[48,288,348] have implicated the phospholipid fraction of milk as the site of oxidative deterioration in fluid milk and cream. This has been substantiated by the studies of Swanson and Sommer[322] and Smith and Dunkley[310] which showed a small but marked decrease in the iodine number of the phospholipids isolated from oxidized fluid milk, whereas those values of butterfat showed insignificant variations. On the other hand, it must be assumed that in products such as butter and dry whole milk both the phospholipid and glyceride fractions of milk can play a significant role in deterioration of the product. The oxidative deterioration of butteroil is undoubtedly a result of glyceride decomposition.

Spontaneous Oxidation in Fluid Milk

Based on the previously referred to observations, it would appear that each dairy product has its own practical as well as theoretical considerations in regard to oxidative deterioration. Possibly in no other product is this exemplified more than in fluid milk on which literally hundreds of scientific endeavours have been undertaken. To illustrate the extent of the problem, Potter and Hankinson[266] reported that 23.1% of 2,955 samples, tasted from individual cows

representing the major breeds, were criticized for oxidized flavor after 24- to 48-hr. storage. Furthermore, numerous investigators have observed substantial increases in the incidence of oxidized flavor in samples contaminated with metallic ions. The latter represents results of an induced type of oxidation which was previously important when copper alloy equipment was in general dairy use. It was for this reason that Thurston[347] classified milks, as follows: (a) spontaneous, for those milks that spontaneously develop the off-flavor within 48 hr. after milking; (b) susceptible, for those milks that, after contamination with cupric ions, will develop the off-flavor within 48 hr.; and (c) resistant, for those milks that will exhibit no flavor, even after contamination with copper and storage for 48 hr.

Experience has shown that certain animals consistently produce milk which may develop off-flavors spontaneously, others occasionally and still others not at all.[247] Of interest in this connection is the observation of Guthrie and Bruechner[117] that the milks from each quarter of an animal differed in their susceptibility to oxidative deterioration. The failure of some milks to develop oxidized flavor in the presence of added copper is a puzzling quality of these milks. Greenbank[109] attributes the resistance of certain milks to its poising action, i.e., the resistance of milk to a change in oxidation-reduction potential.

That a correlation exists between the oxidized flavor and conditions favoring a mild oxidation, as measured by the oxidation-reduction potential, was shown by Tracy *et al.*[352] and by Greenbank.[109] This apparent correlation, as well as other factors, tends to discredit previous theories as to the role of enzymes as catalytic agents in the development of oxidized flavor. Such a theory had been proposed by Kende[181] who claimed milk contained "oleinase" which catalyzed the oxidation of oleic acid to produce the characteristic off-flavor. In recent years, the possible role of xanthine oxidase as a catalytic agent in the development of spontaneously oxidized milk has been fostered by Aurand, Woods, *et al.*[10,11,367] These workers differentiated between spontaneous and nonspontaneous milks on the basis of enzymatic activity. However, the work of Smith and Dunkley[309] did not substantiate these results and they concluded that xanthine oxidase was itself not a limiting factor in the off-flavor.

Despite the lack of complete understanding as to the nature of oxidative deterioration in fluid milk and its products, various factors are known which tend to promote or inhibit the oxidative reaction in dairy products.

Effect of Storage Temperature

Tracy[351] recognized that fluid milk was more susceptible to oxidized flavor when stored at 4° than at 20°C. Bell[17] reported that other conditions being equal, condensed milk stored at −17°C. is more susceptible to the development of an oxidized flavor than is condensed milk maintained at −7°C. In contrast, low storage temperatures tend to inhibit the development of a tallowy flavor in butter and dry whole milk. Pyenson and Tracy[270] reported that storage temperatures of 2°C. retarded the development of oxidative deterioration, as determined by O_2 absorption and flavor scores, in comparison to dry whole milks stored at 38°C. in an atmosphere of air. The results of Holm *et al.*[147] showed that in the case of butter, approximately four times more storage time was necessary at −10°C. to obtain the same two point decrease in flavor score given products held at 10°C. Similar results were obtained by Greenbank *et al.*[115] on these two latter products.

Effect of Oxygen

The inhibition of oxidative deterioration in fluid milk held at higher storage temperatures has been attributed by various workers[48,109,346] to a lowering of the oxygen content as a result of bacterial activity. In this respect, it has been noted that the increase in incidence of oxidized flavor in milk has paralleled the bacteriologically improved milk supply.[153] Collins and Dunkley[38] have reported that although large numbers of bacteria slightly retarded development of oxidized flavor, the relatively small numbers of bacteria normally found in market milk are of no practical importance in determining whether or not milk will develop the off-flavor. Furthermore, Sharp *et al.*[302] stated that the number of bacteria necessary to reduce the oxygen content materially would be sufficient to cause other types of deterioration.

Removal of the dissolved O_2 in fluid milk, or its replacement with N_2, was shown by Dahle and Palmer[48] to inhibit the development of oxidized flavors. Sharp *et al.*[300] further showed that deaeration would inhibit the appearance of the off-flavor even in the presence of 0.1 mg. per liter of copper. Schaffer *et al.*,[292] applying deaeration to products other than fluid milk, concluded that in order to prevent the production of tallowy flavor in butteroil, the amount of available O_2 should be less than 0.8% of the volume of the fat. Similar storage conditions were also proposed by Lea *et al.*[209] Although the deaeration of these two products is of significance only from a scientific

standpoint, the deaeration of dry milk products has practical applications.

Vacuum treatment or replacement of available oxygen with an inert gas has proved its reliability in preventing or retarding the onset of oxidation in dry whole milk for extended periods of storage. Greenbank *et al.*[115] showed that inert gas packing at the 3–4% level increased storage life of whole milk powder two to three times that of air-packed samples, the length of storage being dependent on the initial quality of the product. Lea *et al.*[209] showed that whereas powder packed at the 3–6% oxygen level retarded oxidative deterioration significantly, inert gas containing 0.5–1.0% of O_2 would prevent the development of recognizable tallowy flavors for an indefinite period. Schaffer *et al.*[292] concluded that the time required for the production of a tallowy flavor is inversely proportional to the oxygen concentration.

Several deaeration techniques other than mechanical methods have been utilized to inhibit or retard the development of tallowy flavors in dry milks. Meyer and Jokay[221] reported that milk powders packed in the presence of an O_2 scavenger (glucose oxidase-catalase) and desiccant (calcium oxide) were comparable flavorwise to samples stored in the presence of an inert gas—the enzymes demonstrating the ability to reduce O_2 levels to 0.5% in one week. Jackson and Loo[151] employed an oxygen absorbing mixture (0.5 gm. Na_2SO_3 and 0.75 gm. of $CuSO_4 \cdot 5H_2O$) enclosed in porous paper pouches and demonstrated keeping qualities equal to those of dry milks stored in the presence of an inert gas.

Effect of Heat Treatment

The inhibitory effect of high heat treatment on oxidative deterioration in fluid milk and its products has been reported by various workers.[48,109,117] Gould and Sommer[106] in conjunction with studies on the development of a cooked flavor in heated milks noted a decrease in the oxidation-reduction potential of the product. They attributed the cooked flavor to the formation of sulfhydryl compounds and correlated the liberation of these compounds to the heat retardation and prevention of the oxidized flavor. The work of Josephson and Doan[166] conducted simultaneously with these workers confirmed the relationship between sulfhydryl compounds, cooked flavor, decreased Eh, and inhibition of the oxidized flavor. They further reported that most heated milk products do not become tallowy or oxidized until the sulfhydryls are first oxidized and the cooked flavor has disappeared. In this respect, Gould and Keeney[105] showed that

the oxidized flavor occurred in heated cream to which copper had been added when the active sulfhydryl compounds had decreased to a level approximating 3 mg. per liter of cysteine HCl. β-lactoglobulin has been shown by Larson and Jenness[207] and later confirmed by Hutton and Patton[148] to be the major source of sulfhydryl groups in milk while the fat globule membrane material contributes a minor portion of these reducing compounds.

Time-temperature relationships have been established by various workers as being optimum for preventing or retarding the development of oxidized or tallowy flavors in dairy products; Cream—88°C. for 5 min.[105]; Condensed milk—76.5°C. for 8 min.[17]; dry whole milk—preheat at 76.5°C., for 20 min.[37]; frozen whole milk —76.5°C. for 1 min.[20] Few if any instances of a tallowy flavor have been reported in evaporated milk. Undoubtedly, a major reason for its stability toward oxidation can be attributed to sterilization temperatures employed in its manufacture.

Literature dealing with the inhibitory effect of sulfhydryls on the stability of butterfat obtained from heated butter or cream is conflicting.[69] However, it is of interest to note that Josephson reported[165] that butterfat prepared from butter which was heated to 149°, 177°, and 204.5°C. was extremely stable against oxidation while that heated to 121°C. oxidized readily when stored at 60°C. When butteroil itself was heated from 121° to 204.5°C. it also oxidized rapidly. One per cent skimmilk powder added to butterfat prior to heating at 204.5°C. for 10 min. also exhibited a significant antioxygenic effect which Josephson concluded was the result of a protein-lactose reaction (caramelization).

Effect of Light

The catalytic effect of natural light in promoting off-flavor development in fluid milk has been recognized for a good number of years.[28,121,125] The extent of deterioration appears to be dependent on the wavelengths involved, intensity of the source and the time of exposure.[109,216] Similar results have been reported on butterfat which had been exposed to the action of natural light.[317] In addition to natural light, incandescent or fluorescent lights employed in storage coolers may promote deteriorative reactions,[307] while the development of off-flavors is the limiting factor in the preservation of dairy products by high energy radiation.[51,143] Efforts to inhibit or retard the onset of off-flavors as a result of exposure to light has led to the introduction of doorstep coolers and in certain cases amber colored milk bottles.

Two distinct flavors may develop in milk exposed to sunlight[353]; a burnt, activated or sunlight flavor and a typically oxidized flavor. It is possible that the presence of contradictory statements in the literature regarding deterioration as a result of exposure to light may be attributed to the failure of various investigators to recognize the existence of more than one off-flavor.

The sunlight flavor has been shown[71,251] to originate in the proteins of milk. Specifically, the degradation of the amino acid methionine to methional has been reported[251,256] to be the principal contributor to sunlight flavor. Studies[251,357] have shown that riboflavin plays a significant role in the development of the off-flavor. Although removal of the riboflavin from milk by passing through florisil prevented the development of the sunlight flavor, such treatments did not prevent the development of the oxidized flavor. The latter observation does not agree with the reports of other workers[123,360] which indicate that riboflavin plays a significant role in the oxidative mechanism.

The role of light in promoting the development of oxidized flavors in fluid milk has not been completely resolved. Evidence has been presented which suggests a correlation between the exposure to light and the oxidation of ascorbic acid, the subject of which will be discussed more fully in subsequent sections.

Effect of Metals

Contamination by copper and iron promotes the development of oxidized flavors in dairy products.[76,120,319] Of these two metals, the cupric ion exerts the greater catalytic effect, while the ferrous ion appears to be more influential than the ferric ion.[109] Nickel can also catalyze the development to some extent, but aluminum, tin, zinc, lead, and stainless steel are practically inert in this respect.[110] Manganese has been reported[84,238] to retard or prevent the development of the oxidized flavor in fluid milk. Greenbank,[110] however, reported the metal had no effect whatsoever on flavor development.

As inferred previously, the change to noncorrodible dairy equipment has reduced considerably the incidence of oxidative deteriorations in dairy products. In those cases where prolonged trouble with off-flavors are encountered, the difficulty can usually be traced to the continued use of equipment capable of contaminating the product with metallic ions.[213]

Both copper and iron are normal constituents of milk, and disregarding variations due to individuality and stage of lactation,[183,230] the former is present at average levels of 130 μg per liter of milk and

the latter at 450 μg per liter of milk. In this respect, King and Dunkley[183] based on numerous analyses over a 2.5 year period, concluded that the copper present in milk as it comes from the cow is an important catalyst of spontaneously oxidized milk.

Natural copper and iron exist in milk to a large extent, if not completely, in the form of complexes with protein, and as such are not dialyzable from milk.[185] Copper and iron added to milk, however, are slightly dialyzable. King *et al.*[185] have shown that 10–35% of the natural copper and 20–47% of the natural iron is associated with the fat globule membrane material. In contrast, only 2–3% of copper and negligible amounts of added iron become associated with the fat globule. Similar results were reported by Mulder and Koppejan.[230] Based on these observations, King *et al.*[185] stated that "the observation that no added iron was associated with the fat globule could partially explain the lower catalytic activity of added iron as compared to added copper."

Olson and Brown[237] and Forster *et al.*[79] concluded that proteins and protein hydrolysates form complexes with copper and as such retard or prevent the development of oxidized flavors in fluid milk. These results differ with observations reported by others.[153,323] Olson and Brown[237] further concluded that "the ionization of copper and its destruction of ascorbic acid is closely associated with the development of oxidized flavors. Apparently anything which decreases the ionization of copper will in return retard the destruction of ascorbic acid and in this manner tend to retard oxidized flavor development. As a result, it seems that many individual factors may have a bearing on the susceptibility of nonsusceptible milk to the development of oxidized flavors."

Effect of Ascorbic Acid

That metallic contamination can accelerate the development of oxidative deteriorations in dairy products is evident from the preceding review. However, its presence or absence is not the over-all deciding factor as to whether or not an oxidative-type flavor develops in a dairy product. Olson and Brown[236] among others[84] showed that washed cream (free of ascorbic acid) from susceptible milk did not develop an oxidized flavor when contaminated with copper and stored for three days. Krukovsky and Guthrie[195] and Krukovsky[194] reported that 0.1 ppm of added copper did not promote oxidative flavors in milk or buttermilk depleted of their vitamin C content by quick and complete oxidation of ascorbic acid to dehydroascorbic acid (by the addition of H_2O_2 or photochemically) fol-

lowed by pasteurization. Similarly, Guthrie[116] reported the resistance of ascorbic acid free-milk to oxidation catalyzed by the action of light. Krukovsky and Guthrie[193,195] further showed that the oxidative reaction in ascorbic acid free-milk could be induced again by the addition of ascorbic acid. Accordingly, these workers and others[35] have concluded that ascorbic acid oxidation in fluid milk is an essential link in a chain of reactions resulting in the development of the oxidized flavor. Reports from various investigators seem to verify this conclusion.

Hand *et al.*[123,124] and Dahle and Palmer[48] observed a correlation between the oxidation of ascorbic acid and the development of an oxidized flavor. Vleeschauwer *et al.*[360] noted a correlation between the onset of the off-flavor and the 2,3-diketogulonic acid content. Pont[263] reported that the addition of ascorbic acid to washed cream in the absence of copper promoted the development of an oxidized flavor in that product; such however was not the case in skim and whole milk samples void of metallic contamination. Wright and Greenbank[368] observed an improvement in keeping quality of dry milks fortified with ascorbic acid prior to drying.

That partial oxidation of ascorbic acid stimulates, and complete oxidation inhibits, the development of off-flavors was observed by several workers.[35,195] Noll and Supplee[235] and Hand[122] reported that light, heat, and metallic contamination behave as catalytic agents which accelerate the oxidation of ascorbic acid in the presence of dissolved oxygen, but are seemingly ineffective in the absence of oxygen. Krukovsky[194] noted that atmospheric oxygen is necessary for the oxidation of ascorbic acid.

The behavior of ascorbic acid in the oxidative reaction is anomalous. Previously referred to investigations indicate that concentrations normal to milk (10–20 mg per liter) promote oxidative deteriorations while higher concentrations (50–200 mg per liter) inhibit the development of off-flavors.[34,123,196,236] Chilson[34] explained the behavior as follows: "When ascorbic acid is added to milk, it acts as a reducing agent which oxidizes more readily than the milkfat, therefore either preventing or prolonging the time required for fat oxidation and the development of an oxidized flavor." Krukovsky and Guthrie[196] concluded "the differences between the sample of milk fortified with large amounts of ascorbic acid, in their abilities to resist the reaction which produces the tallowy flavor, can be attributed to the ability of milk to promote ascorbic acid oxidation in the presence of atmospheric oxygen and the continued availability of the latter." In this respect, Bell and Mucha[19] showed that in frozen milk, which

has a longer storage life than market milk, ascorbic acid defers or delays but does not prevent the onset of the defect.

Recently, Krukovsky has shown[194] that the oxidative reaction is initiated more rapidly in milk when the ratio of ascorbic to dehydroascorbic acid is approximately one to one or lower. He states "that an unfavorable proportion of dehydroascorbic acid could not be accumulated if the rate of its oxidation to nonreducible substances surpassed that of ascorbic acid to dehydroascorbic acid. Consequently, the protective influence of ascorbic acid added in large but variable quantities to milk could be attributed to the exhaustion of occluded oxygen prior to the establishment of a favorable equilibrium between these two forms of vitamin C." In this respect, Jenness and Patton[153] state, "the fact that the reduced form of ascorbic acid may establish an equilibrium with the dehydro form indicates a possible role as hydrogen donor and acceptor of importance in lipid deterioration."

It is of interest to note that Jenness and Patton[153] consider ascorbic acid, under normal conditions, to be the principal poising material of milk. In this respect, Greenbank[110] and others[18,29] have shown that the oxidation of ascorbic acid to dehydroascorbic acid is reflected by gradual increases in Eh. Furthermore, Greenbank[110] has attributed the resistance of certain milks to oxidative deterioration a result of their possessing a well poised system.

Effect of Homogenization

Homogenization was recognized in 1933 by Tracy *et al.*[352] to inhibit the development of an oxidized flavor in fluid milk. Subsequently, similar observations have been reported on cream,[332] ice cream,[47] dry whole milk[146] and frozen condensed milk.[17] The inhibitory effect, however, is not absolute. Roadhouse and Henderson[281] found that the pressure required varied with different milks contaminated with the same concentration of cupric ions. The results of Larsen *et al.*[206] indicate that the inhibitory effect of homogenization is dependent on the degree of metallic contamination. Homogenization does not inhibit the development of oxidized flavors in milk exposed to sunlight,[45,358] but in fact tends to increase the susceptibility of such milks.[174] In this respect Guthrie and Krukovsky[119] reported that homogenized milk depleted of its vitamin C content by exposure to sunlight did not develop an oxidized flavor on storage, whereas those milks in which the ascorbic acid was partially oxidized readily deteriorated on subsequent storage.

Various workers have proposed explanations for the inhibitory

ture of homogenization toward oxidative deterioration. Tracy *et* [352] considered the effect to be apparent rather than actual, result-g from changes in the physical consistency of the milk which may er the taste. These workers based their proposal on the observa-n that homogenization has no apparent effect on the Eh of milk. nilar observations have been noted by others.[206] Still others have oposed that the deterioration is retarded as a result of the migra-n of the phospholipids into either the serum phase[349] or interior of e fat globule[192] or general redistribution of the phospholipids in the ilk proper.[113] King proposed[182] that homogenization effects an rreversible change in the structural configuration of the copper-otein complex in such a way that ascorbic acid is no longer able to itiate the formation of lipid free radicals." Tarassuk and Koops[331] ated that "the decrease in concentration of phospholipids and the pper protein complex per unit of newly formed fat globule surface ea appears to be the most important factor, if not the only one, at retards the development of oxidized flavor in homogenized milk." evertheless, it is evident from the literature that homogenization fords a degree of protection against oxidative deteriorations pro-ded excess metallic contamination or undue exposure to light are t operative.

The literature[110,278] appears to be in general agreement that the use green feeds tend to inhibit and dry feeds promote the development oxidized flavors in dairy products. Furthermore, the observa-ns[44,218] that milks produced during the winter months are more sceptible to oxidative deteriorations are reflected, no doubt, in fferences in the feeding practices. Investigations concerned with e constituents of animal feeds of known antioxidant properties und in milk have been centered on the tocopherols. The results, wever, on normal diets as compared to those supplemented with α-copherol are conflicting and therefore inconclusive. Krukov-y[194] suggests that the inhibitory nature of certain feeding practices ay be a result of the secretion of unknown substances, in addition tocopherols, into milk.

iscellaneous Factors

The development of a fishy flavor in butter is well known and its sociation with salted butter made from acid cream was first demon-rated by Rogers in 1909.[285] Cream acidities ranging from 0.20 to 30% appear to represent those levels where flavor development is arginal.[147,286] While the development of fishy flavors in unsalted utter is rarely encountered,[286] Loftus-Hills[140] attributes the rapid

deterioration of salted acid butters to the ability of hydroperoxides to release chlorine from acid sodium chloride solutions and the ability of chlorine to accelerate the oxidation of fats. The development of fishy flavors in butter and its products, however, is not restricted to those products containing added salt. Pont *et al.*[265] induced the development of a fishy flavor in commercial butterfat by the addition of nordihydroguaiaretic acid and citric or lactic acid. The acids alone in butterfat gave oily and rather less defined fishy flavors. Tarassuk *et al.*[332] reported the development of fishy flavors in washed cream adjusted to pH 4.6. At pH 6.6 to 6.7 it was necessary to heat the samples to acquire similar flavor development.

Although not studied extensively, reports on other dairy products suggest that titratable acidity as well as hydrogen ion concentration tend to influence the development of oxidative deteriorations. Anderson[4] found a relationship between the titratable acidity and the development of an oxidized flavor in milk. Furthermore, his results showed that while milks developed an oxidized flavor at a titratable acidity of 0.19%, the deteriorative mechanism was inhibited when the milks were neutralized to acidities of 0.145% or lower. Greenbank[109] found that an increase in pH of 0.1 was sufficient to inhibit the development of the off-flavor for 24 hr. Anderson[4] reported similar results. In addition to fluid milk, Dahle and Folkes[46] attributed the development of oxidized flavors in strawberry ice cream to the presence of copper and the acid content of the fruit.

Early workers[43,321] ascribed the development of fishy flavors in butter to the liberation of trimethylamine from the oxidative decomposition of lecithin. This theory, however, has been discredited by the observations of van der Waarden[361] and more recently by Forss *et al.*[73]

Carbonyl Content of Oxidized Dairy Products

Recent approaches to the problem of oxidized flavors in dairy products have centered on investigations into the carbonyls produced as a result of the oxidative reaction. In general, the C_1 to C_{12} alkanals, C_5 to C_{11} alk-2-enals, and C_6 to C_{11} alk-2,4-dienals have been identified as end products in autoxidized dairy products. Table 45 summarizes the carbonyls resulting from the autoxidation of the phospholipids isolated from butter,[56] butterfat,[52,66,73,75,98] skimmilk,[76,77] washed cream,[74] dry skimmilk[15] and dry whole milk[246] autoxidized under various conditions.

Despite the great similarity in the qualitative carbonyl content of oxidized dairy products, flavor differences are rather apparent. At-

tempts, however, to correlate the off-flavors with specific compounds or groups of compounds are made difficult for several reasons. These include (a) the multitude of compounds produced, (b) differences in threshold values of individual compounds, (c) similarity of flavors imparted by individual aldehydes near threshold, (d) a possible accumulative effect, flavorwise and with regard to threshold values, of mixtures of the same members of a homologous series, (e) the possible existence of compounds or groups of compounds, such as the nonconjugated unsaturated aldehydes, heretofore not identified in oxidized dairy products, and (f) the difficulties involved in adding pure carbonyl compounds to dairy products as a means of evaluating flavor characteristics. In this respect, Day and Lillard[52] concluded that "in view of the large numbers of aldehydes identified from oxidized milkfat, it does not appear feasible to attribute the odor defect to a few specific compounds. Rather, the presence of the complete spectrum of compounds seems necessary for the typical odor associated with oxidized fat."

Nevertheless, recent findings suggest that the preponderance of certain carbonyls or groups of carbonyls are involved in specific off-flavors in oxidized dairy products. Forss *et al.*[76,77] reported that the C_6 to C_{11} 2-enals and C_6 to C_{11} 2,4-dienals, and more specifically 2-octenal, 2-nonenal, 2,4-heptadienal, and 2,4-nonadienal, constitute a basic and characteristic element of copper induced cardboard flavor in skimmilk. The same workers concluded ". . .that while these compounds in milk closely simulate cardboard flavor, the resemblance is not complete, and that the defect contains further subsidiary flavor elements." Bassette and Keeney[15] ascribed the cereal-type flavor in dry skimmilk to a homologous series of saturated aldehydes resulting from lipid oxidation in conjunction with products of the browning reaction. The results of Parks and Patton[246] suggest that saturated and unsaturated aldehydes at levels near threshold may impart an off-flavor suggestive of staleness in dry whole milk. Forss *et al.*[73,74] reported that the fishy flavor in butterfat and washed cream is in reality a mixture of an oily plus an unidentified carbonyl exhibiting a metallic flavor. N-heptanal, n-hexanal, and 2-hexenal were found to be constituents of the oily fraction in washed cream and these three carbonyls plus heptanone-2 were constituents of the oily fraction isolated from fishy butterfat.

Comparative studies by Forss and co-workers[73,75] on the fishy, tallowy and painty flavors of butterfat tend to emphasize the importance of the relative and total carbonyl contents in dairy products afforded different off-flavors. These studies showed that three fac-

tors distinguished painty and tallowy butterfat from fishy flavored butterfat. First, there was a relative increase in the n-heptanal, n-octanal, n-nonanal, heptanone-2, 2-heptenal, and 2-nonenal in the tallowy butterfat and a relative increase in the n-pentanal and the C_5 to C_{10} alk-2-enals in the painty butterfat. Secondly, the metallic compound was present in such small amounts in both the tallowy and painty flavored butterfats as to have no effect on the flavor. Thirdly, the total weight of the volatile carbonyl compounds was about ten times greater in the tallowy and 100 times greater in the painty butterfat than in the fish-flavored butterfat.

El-Negoumy *et al.*[66] suggest that carbonyls in addition to those implicated by Forss *et al.*[73–75] have a more profound influence on specific flavors of butterfat. They speculate the existence of a homologous series of unidentified compounds which are of especial significance in the off-flavors during the initial stages of autoxidation.

Methyl Ketones and Off-Flavors

Methyl ketones containing odd-numbered carbon atoms from C_3 to C_{15} apparently result for the most part from nonoxidative mechanisms in butterfat—despite reports of their presence in oxidized dairy products (see Table 45) and their theorized formation from the dismutation of hydroperoxides.[16]

Various investigations tend to support a nonoxidative type of reaction leading to the formation of these compounds: (a) Patton and Tharp[259] reported their presence in the steam distillate of steam stripped fresh milkfat and the unsaponified matter of fresh milkfat; (b) their absence in oxidized dry whole milk prepared with steam stripped milkfat in contrast to their presence in conventional dry whole milk held under nonoxidative conditions[246]; (c) the identification of methyl ketones in evaporated milk,[366] a product highly resistant to oxidative deterioration; (d) the complete absence of these compounds in other oxidized fats and oils[98]; and (e) preliminary indications that heating butterfat under nonoxidative conditions results in the formation of a definite quantity of methyl ketones.[297] The identification of methyl ketones in oxidized butterfat can best be explained, therefore, as the result of heat treatments employed in the recovery of carbonyls resulting from the autoxidative mechanism.

In addition to heated and stored dairy products, methyl ketones have also been isolated and identified from blue cheese,[248] undoubtedly a by-product of mold action.

The source of methyl ketones in dairy products is unknown. Täufel *et al.*[341] has demonstrated that methyl ketones can be formed

from fatty acids by heat treatment, while Schmalfuss[294] observed the formation of ketones from fatty acids exposed to light. Wong *et al.*[366] postulated that the decarboxylation of β-keto acids, present in milkfat as intermediates of β-oxidation reactions in accordance with Knoop's theory, or as intermediates in the synthesis of milkfat, may serve as the precursors for the formation of methyl ketones. Ellis[65] reported the formation of methyl hexyl ketones in the alkaline hydrolysis of 12-keto oleic acid. Nevertheless, based on previously referred to investigations, it appears that methyl ketones are liberated in dairy products from lipid materials which are peculiar to milkfat.

Lactones and Off-Flavors

The formation of Δ-lactones, in addition to methyl ketones in heated and stored dairy products, is also the result of a nonoxidative mechanism, but of no less importance. Δ-decalactone and Δ-dodecalactone, which impart characteristic flavors at above threshold levels, have been observed in heated butteroil, dried whole milk, dried cream, evaporated milk and pasteurized, homogenized milks.[171,172,253,342] Evidence has been presented by Mattick *et al.*[220] which seems to indicate that 5-hydroxy acids are the precursors of these compounds. They further postulate the existence of the 5-hydroxy acids in butterfat as simple, readily hydrolyzed esters of unknown character.

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